

Cuticular hydrocarbon profiles of *Lycaeides subsolanus* larvae and their attendant ants

Hisashi ÔMURA^{1)*}, Michihito WATANABE²⁾ and Keiichi HONDA¹⁾

¹⁾ Graduate School of Biosphere Science, Hiroshima University, Higashihiroshima, 739-8528 Japan

²⁾ Laboratory of Natural Science for Coexistence of Humans and Nature, Nonprofit Organization Mount Fuji Nature Conservation Center 6603 Funatsu, Fujikawaguchiko, Yamanashi, 401-0301 Japan

Abstract In their late instar stages (third or fourth), *Lycaeides subsolanus* larvae have a facultative symbiotic relationship with several ant species. In Yamanashi Prefecture, the larvae are tended frequently by worker ants of *Lasius japonicus* and sometimes by those of *Formica japonica*. Cuticular hydrocarbons (CHCs) were extracted from wild-caught late-instar larvae of *L. subsolanus* and the workers of these 2 attendant ant species, and their compositions were determined by gas-chromatography mass-spectrometry (GC-MS). The attendant ants showed species-specific CHC compositions; the major compounds obtained from *L. japonicus* were determined to be branched alkanes with >29 carbon atoms, while those from *F. japonica* were determined to be linear alkanes and alkenes with 27 or 29 carbon atoms. The CHC profiles of the third- and fourth-instar larvae were qualitatively similar to each other but remarkably different from those of the attendant ants. The extracts obtained from *L. subsolanus* larvae contained 11 linear and 4 branched alkanes, the major compounds being nonacosane, pentacosane, heptacosane, and hentriacontane. The CHC profile of *L. subsolanus* larvae was quite similar to that of the larvae of *L. argyrognomon*, a closely related species. Our previous studies have shown that the CHC profiles of *L. argyrognomon* larvae were qualitatively consistent irrespective of the larval instar and the attendant ant species. These results indicate that both *L. subsolanus* and *L. argyrognomon* larvae can maintain symbiotic relationships with ants without changing their intrinsic CHC profile.

Key words *Lycaeides subsolanus*, larva, cuticular hydrocarbon, myrmecophily, ant tending.

Introduction

The majority of lycaenid butterflies have obligate or facultative relationships with ants, ranging from parasitic to mutualistic (Pierce *et al.*, 2002). Obligate relationships are observed in particular lycaenid species, in which the larvae obtain ecological benefits from a single ant species that serves as the exclusive attendant. Parasitic relationships, in which the late-instar larvae penetrate into the ant nest and feed on ant broods or regurgitations, are also considered obligate relationships. In such obligate and parasitic relationships, the larvae change their cuticular hydrocarbon (CHC) profiles to chemically resemble the CHC profile of the host ant. This is chemical mimicry by or camouflage of lycaenid butterflies. To date, a few parasitic lycaenid butterflies such as *Maculinea rebeli* and *Niphanda fusca* have been studied for their larval CHC profiles (Akino *et al.*, 1999; Hojo *et al.*, 2009).

Facultative relationships account for almost 50% of the 665 recorded lycaenid-ant relationships (Pierce *et al.*, 2002). Within such relationships, lycaenid larvae establish mutualisms with several ant species; the larvae provide nutrition from dorsal secretory organs in exchange for the protection provided by the ants against predation and

parasitism. Despite the abundance of facultative mutualisms in nature, the signaling mechanism that circumvents ant attacks on the larvae remains poorly understood. Because the larval CHC profiles have been studied in only a few lycaenid species, it remains unclear whether the larvae employ chemical mimicry and camouflage in their facultative relationships with ants.

Lycaeides subsolanus (Eversmann, 1851) is a univoltine lycaenid butterfly inhabiting the midlands of Honshu and Hokkaido in Japan. The mountainous meadows in and around Mt. Fuji are one of the principal habitats of this species. Recently, however, their population has been rapidly declining because of disturbance by human activities and shading by cover plants (Kitahara, 2004). The larvae feed on several fabaceous plants. While the larvae are known to frequently feed on *Vicia unijuga* and *Hedysarum vicioides*, *Vicia pseudo-orobus*, and *Wisteria floribunda* have been newly discovered as the host plants (Watanabe and Miyashita, 2006; Watanabe, 2011). In Yamanashi Prefecture, larvae are tended by workers of several ant species, *Lasius japonicus* being the prominent one (Watanabe and Miyashita, 2006), followed by *Formica japonica* (Watanabe and Miyashita, 2006). The purpose of this study is to examine the CHC compositions of *L.*

*Corresponding author. E-mail: homura@hiroshima-u.ac.jp

subsolanus larvae and 2 species of ant workers and to clarify whether the larvae mimic the CHC profiles of attendant ants in their symbiotic relationship.

Materials and methods

Insects

We collected 24 fourth-instar and 11 third-instar larvae of *L. subsolanus*, and attendant ants belonging to the species *L. japonicus* and *F. japonica* in Fujikawaguchiko Town (Funatsu, Kawaguchi, and Nishikawa regions), Fujiyoshida City (Nashigahara region), and Fuefuki City (Misaka region) in Yamanashi Pref. in May and June in 2009 and 2010.

Sample Preparation

Each individual of larva and the attendant ants ($N = 1\text{--}4$) were frozen to death at $-20\text{ }^{\circ}\text{C}$, and soaked in 1 mL of purified double-distilled dichloromethane for 3 min. The resulting extracts were filtered and concentrated to 50 or 100 μL under a nitrogen stream at $10\text{ }^{\circ}\text{C}$. The concentrated extracts were stored at $-20\text{ }^{\circ}\text{C}$ until chemical analyses.

Chemical Analyses of Cuticular Hydrocarbons

The concentrated extracts were examined by gas chromatography–mass spectrometry (GC–MS). GC–MS analyses were performed at 70 eV using a Shimadzu QP5000 mass spectrometer coupled with a Shimadzu GC-17A gas chromatograph equipped with a J&W Scientific DB-1 fused-silica capillary column (0.25 mm ID \times 15 m, 0.25 μm film thickness), with a temperature program from $50\text{ }^{\circ}\text{C}$ (held initially for 1 min) to $320\text{ }^{\circ}\text{C}$ (held for 5 min) at $10\text{ }^{\circ}\text{C}/\text{min}$. The interface temperature was $320\text{ }^{\circ}\text{C}$. The samples were injected splitless at $320\text{ }^{\circ}\text{C}$ using He as the carrier gas (at a rate of 1.9 mL/min). To calculate the equivalents of chain length (ECLs) for CHC components, a series of authentic *n*-alkane samples were purchased (Tokyo Chemical Industry and Sigma-Aldrich) and analyzed under the same analytical conditions as those used to analyze the extracts. The *n*-alkane components in the samples were identified by comparing their retention data and mass spectra with those of authentic samples, while other hydrocarbon components were tentatively identified on the basis of the ECL data and mass spectra reported in previous studies (Akino *et al.*, 2002; Akino and Yamaoka, 2005; Akino, 2006; Tanigaki *et al.*, 2007). Each CHC component in the extracts of the *L. subsolanus* larvae was quantified using an authentic *n*-alkane with the same carbon number in the principal chain.

Results and Discussion

The workers of the 2 ant species showed species-specific CHC profiles. The CHC profile of *L. japonicus* workers was mainly composed of linear and branched alkanes containing 29 or more carbon atoms (Fig. 1A). Since the total ion chromatograms of the *L. japonicus* extracts in this study were similar to those reported by Akino and Yamaoka (2005), the major components were determined to be monomethyl and dimethyl alkanes. *F. japonica* workers had relatively large amounts of linear alkanes and alkenes with 27 or 29 carbon atoms (Fig. 1B), and the predominant component at the retention time of 20.67 min was determined to be 9-heptacosene (Akino *et al.*, 2002). Interestingly, the CHC profiles of *L. subsolanus* larvae were remarkably different from those of the attendant ants (Fig. 1C, D). The CHC profiles of the third- and fourth-instar lycaenid larvae were qualitatively similar and were comprised of 11 linear and 4 branched alkanes (Table 1). Of the 15 components, nonacosane (C29) was the most predominant and present in amounts of $> 1\text{ }\mu\text{g}$ per larva. Other major components were pentacosane (C25), heptacosane (C27), and hentriacontane (C31). These results indicate that *L. subsolanus* larvae never mimic the CHC profiles of the attendant ants in their facultative relationship. Although a remarkable inter-individual difference was observed in the quantity of each major CHC component, the quantity obtained from fourth-instar larvae was 1.6–3.8 times higher than that obtained from third-instar larvae. Such an inter-instar difference in the quantity of CHCs was probably due to the larval body size.

Our previous study revealed that the major CHC components of *Lycaeides argyrognomon* larvae sharing facultative symbiotic relationships with several ant species are linear alkanes with an odd number of carbon atoms, such as C25, C27, C29, and C31 alkanes (Ômura *et al.*, 2009). The present study clarified that the CHC profiles of the *L. subsolanus* and *L. argyrognomon* larvae are almost the same. Such a similarity indicates a close phylogenetic relationship between the 2 lycaenid species. In addition, *L. argyrognomon* (Ômura *et al.*, 2009) and *L. subsolanus* are highly likely to maintain an intrinsic larval CHC profile irrespective of the attendant ant species. The amount of major CHC components obtained from *L. subsolanus* was approximately twice as large as that obtained from *L. argyrognomon*. This might reflect a difference in body size between the 2 species.

Several parasitic lycaenid species have been studied for their larval CHC profiles before they penetrate into an ant nest. Fourth-instar *M. rebei* larvae not being tended by ants are rich in linear alkanes with an odd number of

carbon atoms, with C31 being the predominant compound (Akino *et al.*, 1999), while second-instar *N. fusca* larvae produce high quantities of methyl-branched CHCs, with 4-methyltriacontane being the predominant component (Hojo *et al.*, 2009). These larvae, when penetrating into ant nests in their late instar stages, modify their CHC profiles to chemically mimic the host ant species (Akino *et al.*, 1999; Nash *et al.*, 2008; Hojo *et al.*, 2009). In contrast, *L. argyrognomon* (Ômura *et al.*, 2009) and *L. subsolanus* larvae, as observed in this study, do not change their CHC profiles, which suggests that these lycaenid species can maintain symbiotic relationships with ants without changing intrinsic CHC profiles. Elmes *et al.* (2002) suggest that the dominance of linear alkanes in their CHC profile renders lycaenid larvae chemically

insignificant and less attractive to worker ants. Further studies are needed to clarify the CHC profiles of other lycaenid larvae and the potential functions of linear alkanes in their facultative relationship with ants.

Acknowledgments

We thank Dr Mamoru Terayama of the University of Tokyo for identification of attendant ant species and the staffs of the Japan Ground Self-Defense Forces (JGSDF), North Fuji Maneuver Area, for granting us permission to conduct our study in the Nashigahara region. This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) to H.O. (No. 22780047).

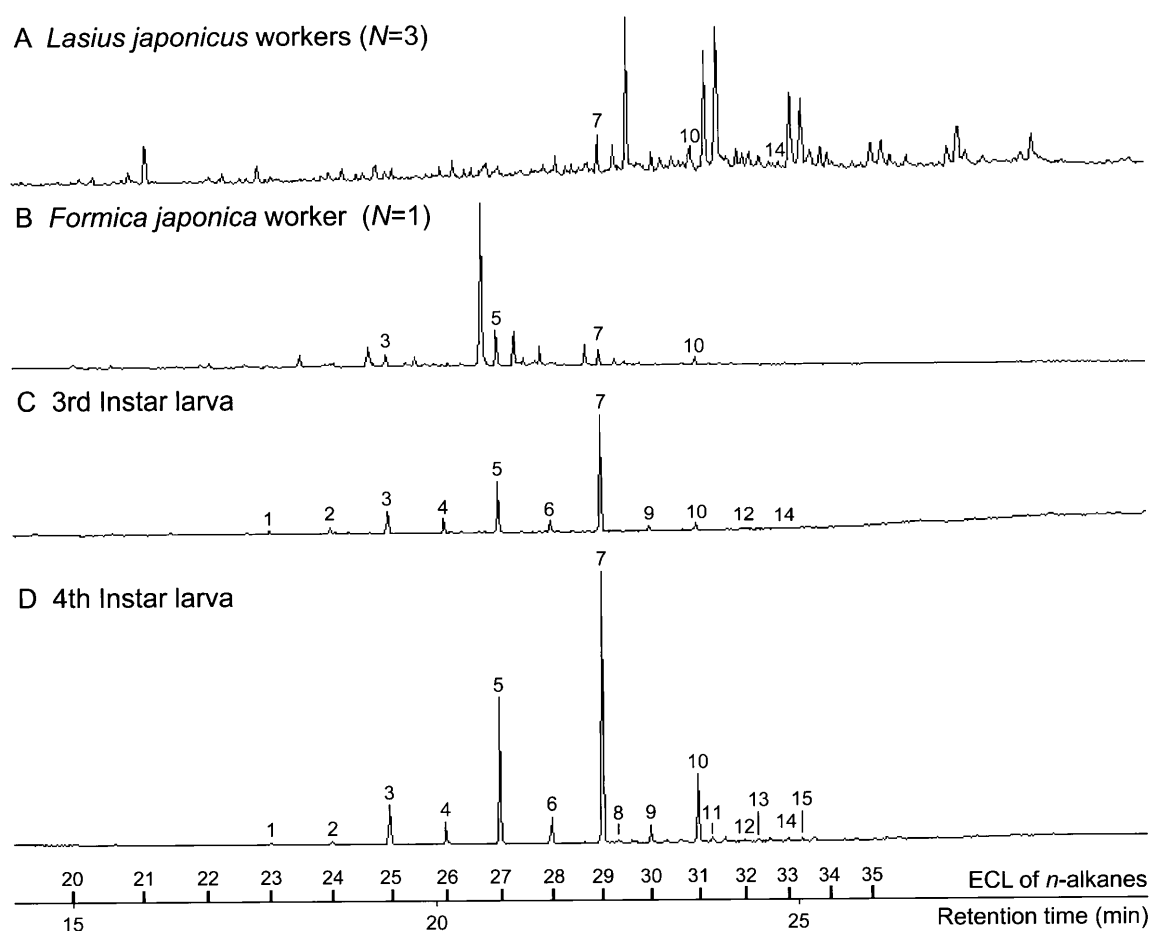


Fig. 1. Typical total ion chromatograms of cuticular compounds from (A) *Lasius japonicus* workers, (B) *Formica japonica* workers, (C) the third-instar *Lycaides subsolanus* larvae, and (D) the fourth-instar *L. subsolanus* larvae. Peaks: 1, tricosane; 2, tetracosane; 3, pentacosane; 4, hexacosane; 5, heptacosane; 6, octacosane; 7, nonacosane; 8, 15/13/11-methylnonacosane; 9, triacontane; 10, hentriacontane; 11, 15/13/11-methylhentriacontane; 12, dotriacontane; 13, 14/12-methyldotriacontane; 14, tritriacontane; 15, 15/13/11-methyltritriacontane.

Table 1. Chemical composition of cuticular hydrocarbon components obtained from *L. subsolanus* larvae.

Peak No.	RT(min)	ECL	Compound	Amount per individual (mean \pm SD) [ng]	
				4th Instar (N=24)	3rd Instar (N=11)
1	17.71	23.00	Tricosane	23 \pm 22	24 \pm 31
2	18.54	24.00	Tetracosane	62 \pm 47	36 \pm 39
3	19.34	25.00	Pentacosane	339 \pm 239	214 \pm 200
4	20.12	26.00	Hexacosane	194 \pm 130	106 \pm 98
5	20.86	27.00	Heptacosane	1035 \pm 698	535 \pm 421
6	21.58	28.00	Octacosane	248 \pm 175	113 \pm 102
7	22.28	29.00	Nonacosane	2736 \pm 1780	1278 \pm 1112
8	22.50	29.32	15/13/11-Methylnonacosane*	32 \pm 27	28 \pm 36
9	22.96	30.00	Triacontane	188 \pm 154	64 \pm 64
10	23.62	31.00	Hentriacontane	787 \pm 634	209 \pm 268
11	23.82	31.32	15/13/11-Methylhentriacontane*	83 \pm 55	24 \pm 34
12	24.24	32.00	Dotriacontane	24 \pm 25	7 \pm 13
13	24.44	32.32	14/12-Methyldotriacontane*	16 \pm 21	19 \pm 31
14	24.86	33.00	Tritriacontane	43 \pm 49	15 \pm 29
15	25.05	33.32	15/13/11-Methyltritriacontane*	38 \pm 38	7 \pm 19

*Tentative identification by comparison of ECL data previously reported.

References

- Akino, T., 2006. Cuticular hydrocarbons of *Formica truncorum* (Hymenoptera: Formicidae): description of new very long chained hydrocarbon components. *Appl. Entomol. Zool.* **41**: 667-677.
- Akino, T., J. J. Knapp, J. A. Thomas and G. W. Elmes, 1999. Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of *Myrmica* ant colonies. *Proc. R. Soc. B* **266**: 1419-1426.
- Akino, T., M. Terayama, S. Wakamura, and R. Yamaoka, 2002. Intraspecific variation of cuticular hydrocarbon composition in *Formica japonica* Motschoulsky (Hymenoptera: Formicidae). *Zool. Sci.* **19**: 1155-1165.
- Akino, T. and R. Yamaoka, 2005. Trail discrimination signal of *Lasius japonicus* (Hymenoptera: Formicidae). *Chemoecology* **15**: 21-30.
- Elmes, G. W., T. Akino, J. A. Thomas, R. T. Clarke and J. J. Knapp, 2002. Interspecific differences in cuticular hydrocarbon profiles of *Myrmica* ants are sufficiently consistent to explain host specificity by *Maculinea* (large blue) butterflies. *Oecologia* **130**: 525-535.
- Hojo, M. K., A. Wada-Katsumata, T. Akino, S. Yamaguchi, M. Ozaki and R. Yamaoka, 2009. Chemical disguise as particular caste of host ants in the ant inquiline parasite *Niphanda fusca* (Lepidoptera: Lycaenidae). *Proc. R. Soc. B* **276**: 551-558.
- Kitahara, M., 2004. Butterfly community composition and conservation in and around a primary woodland of Mount Fuji, central Japan. *Biodivers. Conserv.* **13**: 917-942.
- Nash, D. R., T. D. Als, R. Maile, G. R. Jones and J. J. Boomsma, 2008. A mosaic of chemical coevolution in a large blue butterfly. *Science* **319**: 88-90.
- Ômura, H., Watanabe, M. and K. Honda, 2009. Cuticular hydrocarbons of larva and pupa of Reverdin's blue, *Lycaeides argyrognomon* (Lycaenidae) and its tending ants. *Trans. lepid. Soc. Japan* **60**: 203-210.
- Pierce N. E., M. F. Braby, A. Heath, D. J. Lohman, J. Mathew, D. B. Rand and M. A. Travassos, 2002. The ecology and evolution of ant association in the Lycaenidae (Lepidoptera). *Annu. Rev. Entomol.* **47**: 733-771.
- Tanigaki, T., R. Yamaoka and T. Sota, 2007. The role of cuticular hydrocarbons in mating and conspecific recognition in the closely related longhorn beetles *Pidonia grallatrix* and *P. takechii*. *Zool. Sci.* **24**: 39-45.
- Watanabe, M., 2011. Possibility about the enlargement of larval host plant use in two threatened butterflies, *Eurema laeta* (Pieridae) and *Lycaeides subsolanus* (Lycaenidae). *Yadoriga* **231**: 38-42. (In Japanese)
- Watanabe, M. and Y. Miyashita, 2006. *Vicia pseudo-orobus* as a new larval food plant of *Lycaeides subsolanus* (Lepidoptera, Lycaenidae). *Trans. lepid. Soc. Japan* **57**: 123-126. (In Japanese with English summary)

摘要

アサマシジミ幼虫と随伴アリの体表炭化水素プロファイル (大村 尚・渡邊通人・本田計一)

アサマシジミ老齢 (3~4 齢) 幼虫は数種のアリと任意の共生関係を築いており、山梨県ではトビイロケアリやクロヤマアリ等の随伴が観察される。野外採集したアサマシジミ 3・4 齢幼虫とこれら随伴アリのワーカーから体表炭化水素 (CHC) を抽出し、GC-MS でその化学組成を調べた。2 種の随伴アリは種特異的な CHC 組成を示し、トビイロケアリの主要成分は炭素数 29 以上の分枝アルカンであるのに

対して、クロヤマアリの主要成分は炭素数27・29の直鎖アルカンおよびアルケンであった。アサマシジミ幼虫のCHC組成は3齢および4齢幼虫で互いに酷似していたが、随伴アリのそれとは著しく異っていた。幼虫抽出物は11種類の直鎖アルカンと4種類の分枝アルカンを含んでおり、その主要成分は nonacosane, pentacosane, heptacosane, hentriacontane であった。アサマシジミ幼虫は近縁のミヤマシジミ幼虫と類似のCHCプロフィールを示した。先行研究

において、ミヤマシジミ幼虫のCHC組成は幼虫の齢や随伴アリの種類に応じて変化しないことを見いだしている。これらの事実から、アサマシジミ幼虫もミヤマシジミ幼虫と同様に生得的なCHC組成を維持したままアリとの共生関係を構築していると考えられる。

(Received April 9, 2012. Accepted September 10, 2012)